Fundam. appl. Nematol., 1998, **21** (4), 319-326

Variability among four populations of *Meloidogyne javanica* from Brazil

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Accepted for publication 13 August 1997.

Summary – The morphology, isozyme profiles, karyology, host ranges, and DNA analysis (PCR-RAPD) of four atypical Brazilian populations (P1-P4) of *Meloidogyne javanica* were compared to those of two standard isolates (S1, S2). The variability of certain morphological characters was correlated with cytogenetic and DNA analysis. These studies permitted the separation of the *M. javanica* populations into two groups: the first includes populations P1, S1, and S2 and the second includes populations P3 and P4. Based on DNA analysis, P2 holds an intermediate topological position, but it was very close to P1 based on morphology and karyology. However, it was possible to separate P1 from P2 by their esterase phenotype and by response on differential hosts. PCR-RAPD permitted detection of intraspecific variability. This is a clear advantage over isozyme and differential host plants in that the number of available enzymatic systems and hosts are limited. Compared to existing reports, our study revealed an unusually high level of variability among populations of *M. javanica*, a species formerly considered to be relatively homogeneous. © Orstom/Elsevier, Paris

Résumé – ***Variabilité chez quatre populations de* Meloidogyne javanica *provenant du Brésil*** – La morphologie, les profils isoenzymatiques, la caryologie, la gamme d'hôtes et l'analyse de l' ADN (PCR-RAPD) de quatre populations atypiques de *Meloidogyne javanica* originaires du Brésil (P1-P4) ont été comparés à ceux de deux populations typiques appartenant à la même espèce (S1 et S2). Ces approches ont permis la séparation de ces populations en deux groupes : P1, S1 et S2, d'une part et P3 et P4, d'autre part. En se basant sur l'analyse de l'ADN, la population P2 présente une position intermédiaire, mais semble très proche de P1 d'après ses caractères morphologiques et le nombre de ses chromosomes. Elle en diffère cependant par son phénotype estérasique et par sa gamme d'hôtes. La technique de PCR-RAPD a permis la détection d'une variabilité intraspécifique notable. Cela représente un avantage certain par rapport à l'utilisation des isoenzymes et des gammes d'hôtes, dans la mesure où le nombre d'hôtes différentiels et de systèmes enzymatiques disponibles est limité. Par comparaison avec d'autres études, nos résultats mettent en évidence une variabilité intraspécifique élevée chez *M. javanica*, espèce considérée jusque là comme relativement homogène. © Orstom/Elsevier, Paris

Keywords: cytogenetics, differential host test, intraspecific variability, isoenzymes, *Meloidogyne javanica*, morphology, nematodes, RAPD-PCR.

Meloidogyne javanica is one of the most common and economically important root-knot nematodes in Brazil (Carneiro *et al.*, 1996). *M. javanica* populations collected from Rio Grande do Sul (RS) and Paraná (PR) States differ in isozyme phenotypes and virulence to differential host plants (Carneiro *et al.*, 1996). One *M. javanica* population from RS showed a high level of virulence against soybean and another one from PR reproduced very well on corn in the field (unpubl.). Three Brazilian isolates of *M. javanica* were compared on soybean under greenhouse conditions: the isolate from the Itamarati farm, Ponta Porã, Mato Grosso do Sul, induced four to five times more galls and eggs on soybean cultivar UFV-4 than the other two isolates. All isolates were typical of *M. javanica* with respect to perineal patterns with distinct lateral incisures and responses on differential host plants (Tihohod & Ferraz, 1986). Based on these results it is clear that an evaluation of the intraspecific variability among these Brazilian *M. javanica* isolates was needed.

Our objective was to use morphological, biochemical, cytogenetic, differential host, and DNA (PCR RAPD) procedures for a comparative study of four atypical Brazilian isolates of *M. javanica* with two standard isolates from INRA-Antibes collection previously studied by Castagnone-Sereno *et al.* (1994).

Materials and methods

NEMATODE POPULATIONS

Each nematode isolate used in this study originated from a single egg mass derived from a field population (Table 1). The isolates were maintained on tomato cv.

Rutgers under greenhouse conditions. Second-stage juveniles (J2) and males were extracted by placing roots containing egg masses in water, aerating them with an aquarium pump, and periodically collecting the nematodes from the water. Females were hand picked from infected tomato roots.

MORPHOMETRIC STUDIES

Males and J2 were killed in hot water, transferred to 2% formalin, and measured immediately under light microscopy (LM). Perineal patterns were cut from live young females in 45% lactic acid and mounted in glycerin (Taylor & Netscher, 1974). Males, J2, and females were prepared for scanning electron microscopy (SEM) according to previously described methods (Eisenback & Hirschmann, 1979, 1980; Eisenback *et al.*, 1980). Specimens were viewed and photographed with a JEOL JSM 6301F SEM. At least 50 specimens of each life stage were examined.

BIOCHEMICAL AND BIOLOGICAL STUDIES

Electrophoresis was performed on 7% polycrylamide gel slabs (Carneiro *et al.*, 1996). Cytogenetic studies were made with a propionic orcein staining method (Triantaphyllou, 1985). The differential host test was performed with the following plants: cotton cv. Deltapine 61, tobacco cv. NC 95, pepper cv. California Early Wonder, watermelon cv. Charleston Gray, peanut cv. Florunner, and tomato cv. Rutgers (Hartman & Sasser, 1985).

RAPD PROCEDURE AND ANALYSIS

For each isolate, total genomic DNA was purified from 100 µl of J2. DNA purification, PCR-RAPD, and electrophoretic analysis were performed as described previously (Castagnone-Sereno *et al.*, 1994). Three random primers, purchased from Eurogentec, were used (primer 2: 5′-ATGGATCCGC-3′; primer 20: 5′-TGACCCTCCAAGAAGGT-3′, and primer 41A: 5′-CCCTGGACGTCTACAAT-3′). Reactions were repeated at least three times and a negative control without DNA was included in all assays.

From the whole set of RAPD fragments generated in the *M. javanica* isolates using the different primers, only reproducible bands were considered for further analysis. Similarity coefficients and genetic distances for the pairwise comparisons of the six geographic isolates were calculated as described in Castagnone-Sereno *et al.* (1993). A dendrogram was then generated according to the unweighted pair grouping using the arithmetic averages (UPGMA) method (Sokal & Sneath, 1963) performed with SAS software.

Results

MORPHOLOGY

No major morphometrical variations were observed between the four isolates of *M. javanica* from Brazil. Only useful, stable, morphometric measurements with a CV below 10% (Rammah & Hirschmann, 1990) were used in this paper for characterization. Means and standard error of means of every morphometric character of three life stages (female, male, and J2) were similar among the isolates (Table 2). Qualitative characters such as female and male head and stylet morphology varied only slightly. Distinguishing characters will be reported below.

Female

The perineal patterns were typical for *M. javanica* with a rounded to flattened dorsal arch and distinct lateral lines that clearly delineated the dorsal and ventral region of the patterns (Fig. 1-C).

Stylet robust and stylet cone distinctly curved dorsally in isolates P3 and P4 (Fig. 2 C, D) but only slightly curved in isolates P1 and P2 (Fig. 2 A, B).

Table 1. *Main characteristics of the* Meloidogyne javanica *populations used in this study.*

Population code	Origin	Crops	Esterase phenotypes*	Differential hosts**		Chromosome numbers
				pepper	peanut	
P1	Paraná, Brazil	tomato	J2	+	–	2n = 46-47
P2	Paraná, Brazil	corn	J3	–	–	2n = 46-47
P3	Mato Grosso do Sul, Brazil	soybean	J3	–	–	2n = 42-43
P4	Rio Grande do Sul, Brazil	soybean	J3	–	–	2n = 42-43
S1	La Réunion, France	tomato	J3	–	–	2n = 46-47
S2	Oualidia, Morocco	tomato	J3	–	–	2n = 46-47

* Esterase phenotypes were designated as described by Esbenshade and Triantaphyllou (1985).
** Pepper cv. California Wonder, Peanut cv. Florunner; (+) susceptible host, (–) resistant host.

Table 2. *Morphometric comparison of females, males, and juveniles (J2) of four Brazilian populations of* Meloidogyne javanica *(n = 30, all measurements in µm).*

	Paraná 1 (P1)	Paraná 2 (P2)	Mato Grosso do Sul (P3)	Rio Grande do Sul (P4)
FEMALES				
Stylet	15.4 ± 0.2	15.7 ± 0.1	16.2 ± 0.2	16.0 ± 0.2
	(14.5-17.3)	(15.4-17.2)	(15.1-17.6)	(15.3-17.8)
Stylet knob height	2.0 ± 0.03	2.0 ± 0.03	2.1 ± 0.03	2.0 ± 0.03
	(1.7-2.1)	(1.8-2.4)	(1.8-2.4)	(1.7-2.2)
Stylet knob width	4.6 ± 0.08	4.5 ± 0.06	4.9 ± 0.08	4.8 ± 0.06
	(4.0-5.20)	(3.9-5.4)	(4.2-5.5)	(4.2-5.3)
Stylet knob width/height	2.5 ± 0.06	2.4 ± 0.04	2.5 ± 0.03	2.5 ± 0.04
	(2.2-2.8)	(2.1-2.7)	(2.3-2.9)	(2.3-2.8)
MALES				
Body width at stylet knob	21.2 ± 0.17	19.6 ± 0.18	20.2 ± 0.16	19.8 ± 0.15
	(19.0-22.1)	(17.5-22.2)	(18.4-22.1)	(17.8-21.1)
Stylet	21.4 ± 0.22	21.6 ± 0.10	21.8 ±0.14	21.7 ± 0.18
	(19.3-22.6)	(19.2-22.2)	(19.7-23.7)	(18.8-23.4)
Stylet knob height	2.5 ± 0.04	2.5 ± 0.06	2.6 ± 0.03	2.7 ± 0.04
	(2.4-2.9)	(2.3-2.8)	(2.4-3.1)	(2.2-3.2)
Spicule length	28.3 ± 0.36	28.4 ±0.47	29.6 ± 0.47	30.2 ± 0.22
	(23.2-32.8)	(22.6-34.8)	(22.7-35.4)	(25.4-35.2)
J2				
Stylet	11.6 ± 0.05	11.8 ± 0.04	11.6 ± 0.06	11.5 ± 0.03
	(11.2-12.6)	(11.3-12.4)	(11.3-12.1)	(11.1-11.9)
Stylet base to head end	14.8 ± 0.08	15.1 ± 0.05	14.9 ± 0.03	14.8 ± 0.11
	(13.9-15.8)	(14.9-15.6)	(13.7-15.6)	(13.3-15.9)
Excretory pore to head end	83.4 ± 0.43	82.9 ± 0.97	83.1 ± 0.47	83.7 ± 0.23
	(80.0-90.1)	(64.0-84.9)	(75.4-88.8)	(61.8-89.2)

Shaft cylindrical and broad near the base. Knobs transversely elongate, set off from the shaft, and sometimes marked anteriorly by a shallow indentation (Fig. 2 A-D).

In face view (SEM), the four isolates had labial disc and medial lips dumb-bell shaped (Fig. 1 A). The labial disc had two prominent bumps ventrally. Usually the medial lips were indented, which suggests a division of the lips into pairs of medial lips. The lateral lips were large, elongate, and set off from the medial lips and head region.

Male

In LM, head morphology was consistent among the isolates and typical of *M. javanica*: the labial disc and medial lips form one smooth continuous head cap that is high and rounded and distinctly set off from the head region. In face view (SEM), the large smooth labial disc and rectangular medial lips were fused; the cephalic characters were different among the isolates: medial lips with rounded corners and sometimes with slight indentation at junctions with labial disc in P1 (Fig. 3 A, B) and P2 (Fig. 3 C, D), with more pronounced indentations dividing the medial lips into distinct lip pairs in P3 (Fig. 3 E, F) and P4 (Fig. 3 G, H). Small short annulations were often present (94%) on head region of isolates P3 and P4 (Fig. 3 E-H) and were less frequent (32%) in populations P1 and P2 (Fig. 3 A-D). Stylet and spicule shapes were similar and sizes relatively stable (Table 2).

Isolates P3 and P4 produced 30% of male intersexes. These intersexes showed different degrees of female secondary sex characters, varying from a small ventral protuberance anterior to the spicules to a large protuberance marked by a rudimentary vulva (Fig. 1 D). Isolates P1 and P2 produced only normal males.

Second-stage juvenile

The head morphology was similar among isolates and generally characterized in face view (SEM) by a dumb-bell-shaped head cap (Fig. 1 B). The tail was typically long with narrow tapering tail terminus ending in a finely rounded tip as earlier described for *M. javanica.*

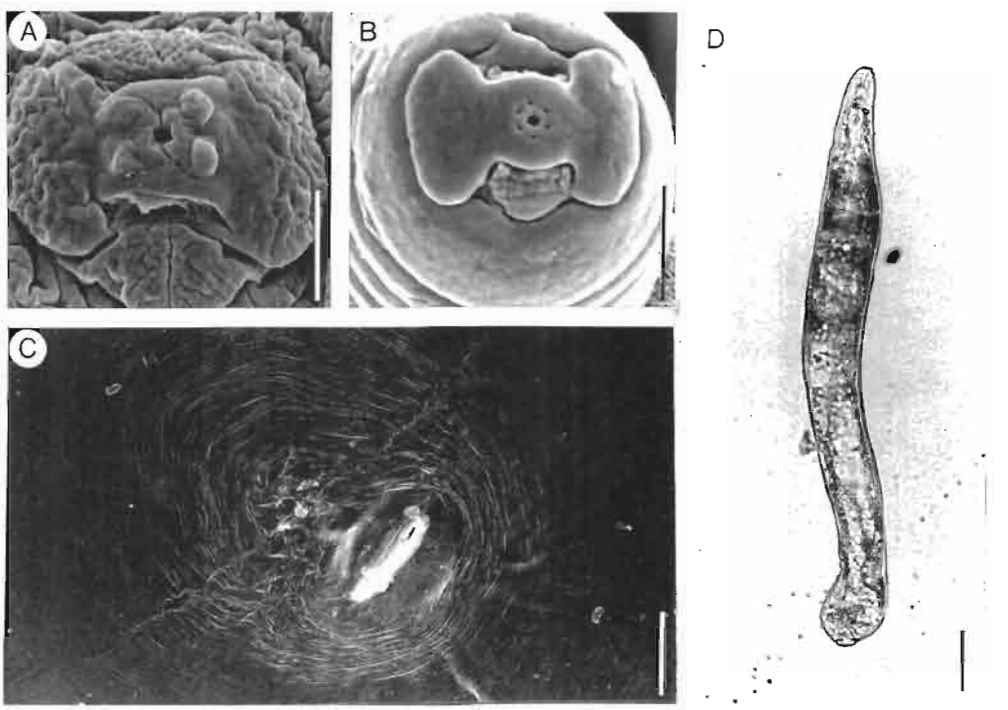

Fig. 1. *SEM photographs of* Meloidogyne javanica, *population P3. A: Face view of female; B: Face view of J2; C: Perineal pattern. LM photographs of* M. javanica, *isolate P3; D: Intersex male (Scale bars: A = 1.64 µm; B = 1.38 µm; C = 20 µm; D = 62 µm; population codes are given in Table 1).*

Biochemistry

Three of the four Brazilian populations (P2, P3, P4) and the standard isolates (S1, S2) had three esterase isozymes detected on polyacrylamide gels and were phenotypically identical to *M. javanica*. Isolate P1 had only two esterase isozymes; these individuals lacked the fastest migrating isozyme reported present in most *M. javanica* individuals (Table 1).

Differential host plants

The P2, P3, and P4 isolates reproduced on tobacco, watermelon, and tomato; they did not reproduce on pepper and peanut (Table 1). The P1 isolate reproduced on pepper, tobacco, watermelon, and tomato.

Cytogenetics

Isolates of *M. javanica* reproduced exclusively by mitotic parthenogenesis. The chromosome number of the six isolates are presented in Table 1.

RAPD analysis

Depending on the nematode DNA and primer combination, five to eight reproducible bands were detected in the 0.1-1.8 kb size range. Fig. 4 shows a typical example of RAPD banding patterns obtained for the six isolates. Of the three primers used, a total of 38 scorable markers was detected, with ten to sixteen loci per primer. Whatever primer was used, two groups could be clearly distinguished among the isolates based on the patterns they displayed: isolates S1, S2, and P1 were distinct from isolates P3 and P4. Position of isolate P2 appeared less clear, as it shared bands with either group depending on the primer used.

Similarity coefficients and genetic distances for the pairwise comparisons of the six isolates were analysed with UPGMA and the resulting phenogram is shown in Fig. 5.

As expected, isolates S1, S2, and P1 clustered on one side of the tree, while isolates P3 and P4 clustered on the other side. Isolate P2 took an intermediate position, with a loose relationship with the first cluster. Without considering the P2 isolate, similarity between the two main clusters is low, which suggests a significant genetic divergence between them.

Discussion

Accurate identification of species and populations is essential for the design of an efficient control plan based on resistance and crop rotation. *M. javanica* can

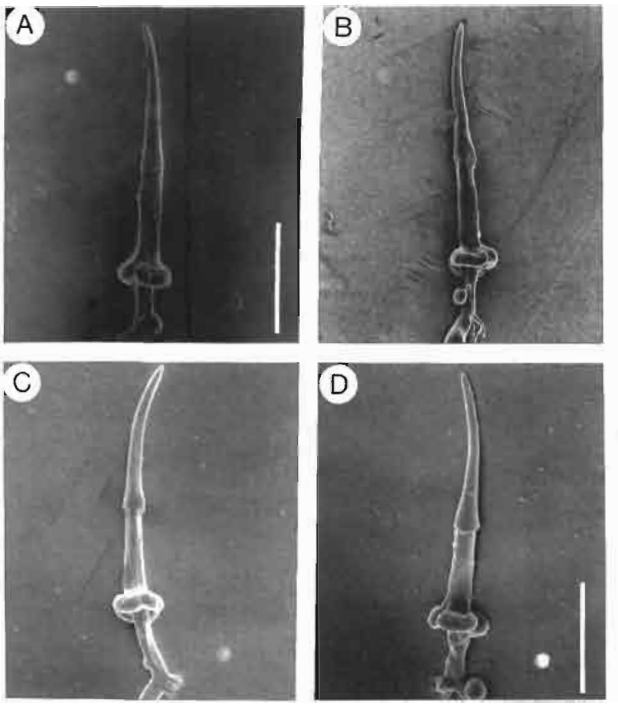

Fig. 2. *SEM photographs of excised stylets of females of* Meloidogyne javanica. *A: Population P1; B: Population P2; C: Population P3; D: Population P4. (Scale bar: A-C = 7.20 µm; D = 6 µm; population codes are given in Table 1).*

be identified reliably based on morphology (Eisenback *et al.*, 1980), biochemical methods (Esbenshade & Triantaphyllou, 1985), and cytogenetic methods (Triantaphyllou, 1985). However, these techniques do not distinguish virulence or race differences within the species (Janati *et al.*, 1982; Rammah & Hirschmann, 1990). Qualitative characters have been shown to be more useful in species determination of *Meloidogyne* than measurements (Eisenback *et al.*, 1981; Hirschmann, 1985; Jepson, 1983*a*, *b*, *c*, 1987; Rammah & Hirschman, 1990). A combination of characters from females, males, and J2 gives reliable identification (Eisenback *et al.*, 1980, 1981; Hirschmann, 1985; Jepson, 1987). Stylet morphology of females and males, head shape of males, and perineal patterns of females have been suggested as the most stable and reliable characters for identification of *M. javanica* populations (Rammah & Hirschmann, 1990). However, Netscher (1978) considers that the high variability of perineal patterns makes these features unreliable for species identification. Nevertheless, the patterns of the isolates in our study had distinct lateral lines, characteristic of *M. javanica.*

The stylet of *M. javanica* has been reported to have a slightly curved cone and wide, low knobs, often indented anteriorly (Eisenback *et al.*, 1981). In our

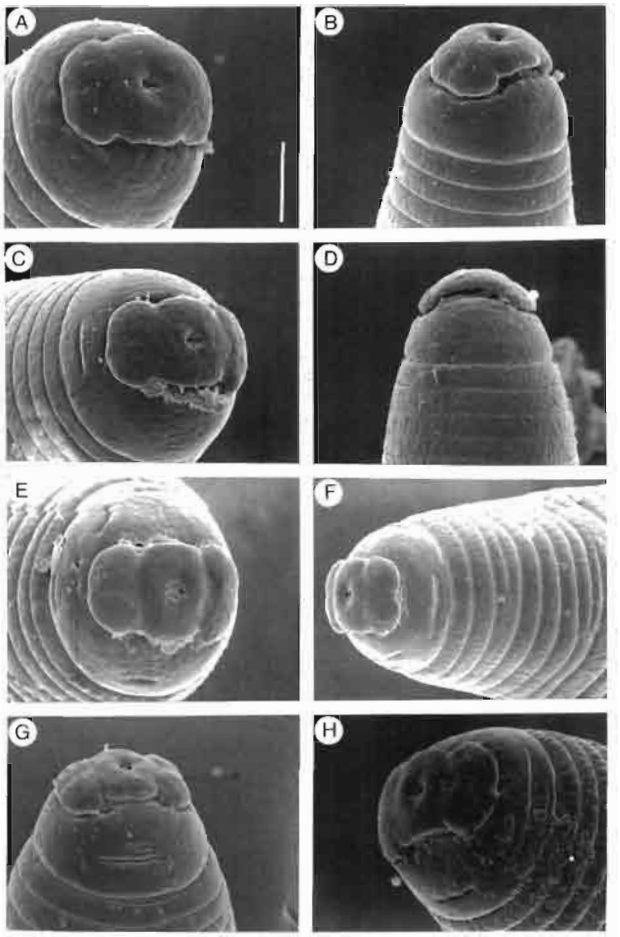

Fig. 3. *SEM photographs of head regions of males of* Meloidogyne javanica. *A, B: Population P1; C, D: Population P2; E, F: Population P3; G-H: Population P4. (Scale bar = 4.5 µm; population codes are given in Table 1).*

SEM studies, the dorsal curvature of the cone was more pronounced on P3 and P4 and slightly pronounced on P1 and P2. Variability of stylet morphology also has been observed among *M. javanica* isolates (Rammah & Hirshmann, 1990; Jepson, 1983*b*). In face view (SEM), the females were typical of *M. javanica.*

Head shape and morphology of males are of great practical value in the identification of the four most common species of *Meloidogyne* (Eisenback & Hirschmann, 1980; Eisenback *et al.*, 1981). In our LM studies, head shape and stylet morphology of males were stable among the four Brazilian isolates and have been considered a species-specific characteristic of *M. javanica* populations (Rammah & Hirshmann, 1990). Our SEM studies of P1 and P2 showed the cephalic characters to be relatively stable and

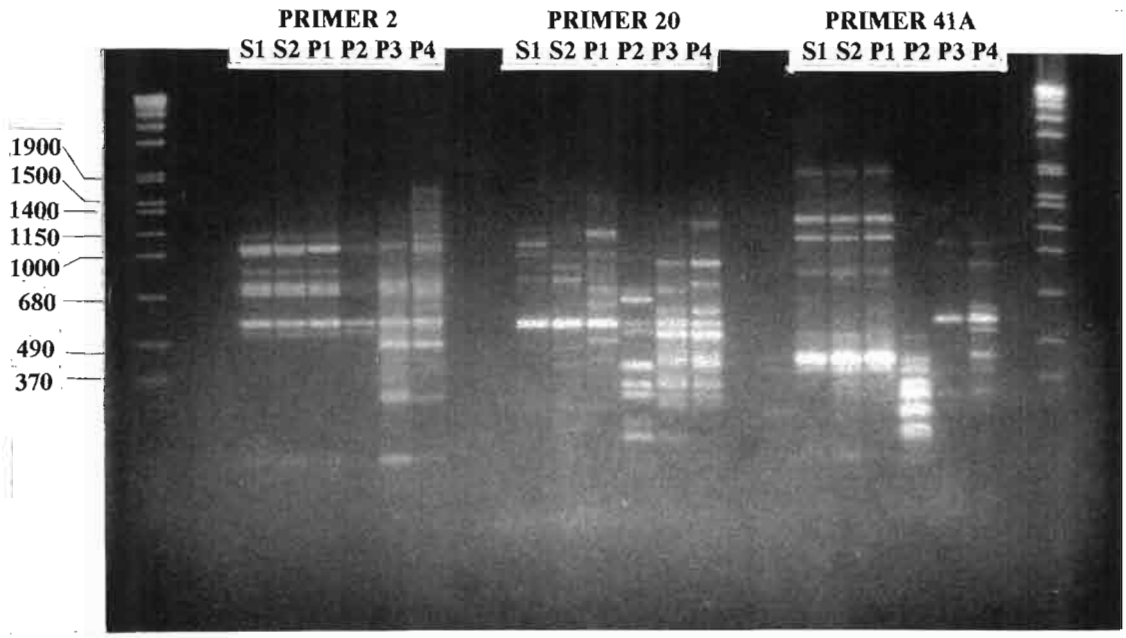

Fig. 4. *RAPD patterns of the six* Meloidogyne javanica *populations tested obtained with primers 2, 20 and 41A. (Population codes are given in Table 1; sizes of molecular weight markers are in base pairs).*

consistent with previous observations (Eisenback & Hirschmann, 1980; Eisenback *et al.*, 1981) for isolates P1 and P2 (Fig. 3 A-D), but the labial disc and medial lips of P3 and P4 were divided by pronounced indentations (Fig. 3 E-H). Variability on male face view of *M. javanica* was observed first by Rammah and Hirschmann (1990).

Our study showed that J2 did not exhibit any useful differentiating characters among the four Brazilian isolates. These results agree with the observations made by Rammah and Hirschmann (1990) who considered that the J2 characters were not diagnostic for *M. javanica*.

The use of a differential host test to identify *M. javanica* isolates proved to be of limited value and esterase profiles provided insufficient information to separate isolates within the species. Using these approaches, only the isolate P1 (race pepper and esterase phenotype J2) could be differentiated. Some populations of *M. javanica* were found to attack pepper or peanut, which are not normally hosts of *M. javanica* (Ogbuji, 1981; Tomaszewski *et al.*, 1994). The J2 esterase phenotype of *M. javanica* was detected by Tomaszewski *et al.* (1994) and Carneiro *et al.* (1996).

Although, some variability was observed in P2, P3, and P4 under field or greenhouse conditions, these three isolates were typical of *M. javanica* in differential host test and esterase phenotypes (Table 1). Intraspecific variation at the enzyme level in *Meloidogyne* spp. is generally low. This is supported by the fact that enzymes are produced via the expression of genes often highly conserved between closely related taxa and representing only a minor fraction of the

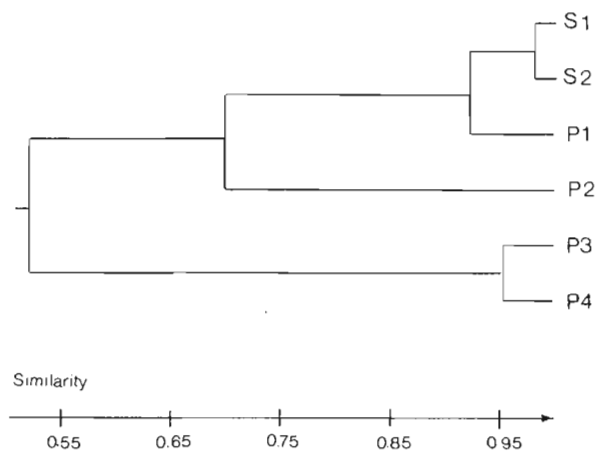

Fig. 5. *UPGMA dendrogram (based on RAPD data) showing the relationships between the six* Meloidogyne javanica *populations tested (Population codes are given in Table 1).*

total genome, whereas non-coding regions are more abundant and subjected to extensive evolutionary changes (McLain *et al.*, 1987).

The chromosome number (Table 1) permitted the differentiation of two types for *M. javanica*: P1, P2, S1, and S2 with 46-47 chromosomes and P3 and P4 with 42-43. This variability was first reported by Janati *et al.* (1982) and Triantaphyllou (1985).

PCR-RAPD procedure and analysis allowed the separation of the six isolates of *M. javanica* into two main clusters: S1, S2, P1 and P3, P4 (Fig. 5). These results agree with the morphological and cytogenetical characterization (Figs 2-5, Table 1). Isolate P2 holds an intermediate topological position (Fig. 5) somewhat closer to the first cluster. Based on morphological and cytogenetical studies, this isolate is similar to isolate P1, but P1 and P2 can be differentiated by esterase (J2) and differential host plants (Table 1).

The results showed the ability of primers using 17-30 nucleotides to detect polymorphisms within *M. javanica* isolates. Although genetic variability was not observed previously among isolates of *M. javanica* (Cenis, 1993; Castagnone-Sereno *et al.*, 1994), these authors argued that because of the small sample size used in their studies, the species-specific or isolate-specific patterns observed might no longer be significant if more isolates were analysed within this species.

PCR-RAPD was the best technique for differentiating intraspecific genetic variation and may be considered a useful tool for diagnosis and for addressing many unsolved questions of variation among species-specific isolates of root-knot nematodes. Nevertheless, genomic DNA alone cannot achieve a true identification of *M. javanica* populations. Our results support the comment made by Hyman (1996) who rejected the use of molecular responses alone for determining phylogenetic affinities among nematodes at any taxonomic level.

In conclusion, it was possible to detect variability within *M. javanica* using morphological, cytogenetical, biochemical, differential host response, and molecular approaches. Morphological differences (detected only using SEM), chromosomes number, and molecular analyses permitted the separation of two subspecific main clusters of *M. javanica* Brazilian isolates. From that point of view, it is clear that more extensive sampling of isolates from world-wide locations is required to strengthen the informative value of the specific and/or subspecific clusters of *M. javanica* populations.

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